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14. ABSTRACT

Our overarching scientific hypothesis holds that serotonergic influences on brain development driven by genetics and early experience induce a variation of normal brain anatomy that makes the brain highly susceptible to the effects of severe stress. Previous findings from our lab support the existence of an anatomical phenotype related to the 5HTTLPR serotonin transporter genetic variant that confers susceptibility to depression, and the current work seeks to extend these findings to PTSD. Initial animal studies confirmed that this serotonin phenotype may affect fear-related behavior by altering neurotransmission in subcortical circuits that include the amygdala. Low serotonin tone was associated with accentuated fear behavior, and we determined that glutamate neurotrtansmission in the basolateral amygdal was a critical element of this response. Preliminary studies support reduced serotonin fiber density in the human thalamus (upstream of the amygdala) in 5HTTLPR-s carriers, consitant with our animal study. In another important region immediately upstream from the amygdala, the medio-orbital prefrontal cortex, we have observed signs of accelerated aging in PTSD, and confimed that consistent with animal models, there is evidence for a reduction in dendritic spine density in PTSD. These findings are among the first post-mortem studies of PTSD ever published. In addition to these scientific findings, our consortium is currently studying over 50 PTSD and similar numbers of MDD and control brains, including 40 brains collected with funding from this project. These brains are now linked with the National Center for PTSD Bank, which has great potential to contribute to future research efforts to understand the pathophysiology of PTSD and other stress-related disorders.

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INTRODUCTION:

Initially, this project in its revised form included an animal model, a longitudinal human study and an human MRI component. Animal work was partially completed before the intial rebudgeting at the recommendation of the steering committee (Section A, below). Local (BAMC) IRB approval was obtained to initiate the longitudinal study. However, HRPO approval was delayed and design flaws in the the MRI magnet forced us to return the equipment to the factory for a redesign and rebuild. These factors made completion of these aims before the end of the funding cycle impossible, and a final revision included only post-mortem work and increased brain collection. Because of our success in brain collection and in working with other brain banks to initiate PTSD brain collection, we can now study over 50 PTSD cases going forward. Post-mortem research is continuing without interruption through 2016 with implementation of the phase II contract, when the main body of the data will be available for analysis and publication.

Our overarching scientific hypothesis holds that serotonergic influences on brain development driven by genetics and early experience induces a variation of normal brain anatomy that makes the brain highly susceptible to the effects of severe stress. Previous work has identified an effect of the 5HTTLPR genetic variation in the serotonin transporter on the anatomy of pulvinar nucleus of the thalamus (Young et al; 2007 PMID: 17083920), which projects to the amygdala and plays an important role in fear responses, and we have also identified a strong influence of this genetic variant on susceptibility to PTSD in combat veterans (Kimbrel et al., 2015 PMID: 25314020). The present report outlines our animal model, which supports a role of low levels of serotonin in the basolateral nucleus of the amygdala (Section A) on fear-related behavior. Our studies report below a 50% reduction of serotonin fiber density in the human thalamus upstream of the amygdala in human 5HTTLPR-s carriers (Section D), consitant with this animal model. We found that the 5HTTLPR-s allele was associated with epigenetic alterations in specific microRNA (miR) populations in the thalamus as well (Section D). Initially, only major depression and control human brain material was to be studied in this grant. Due to a recommendation from the steering committee, this was changed in the middle of the grant period to focus on collection of PTSD brains, and

the grant provided substantial support for the initiation of very successful PTSD brain collection in the later half of the funding period (Section B). This effort has resulted in one of the first published manuscripts on post-mortem PTSD (Young et al., 2015), which identified reduced dendritic spine density in an area of PTSD frontal cortex (Section C). Finally, in this same region of the limbic cortex, we observed signs of accelerated aging/premature senscence in post-mortem PTSD cases (Section C). Signs of premature senescence, loss of interneuron function, and evidence for activated astrocyte activity in the cortex are supported by animal models of stress effects and in human PTSD studies by observations of advanced aging in blood and other organ systems. As we proceed with molecular and anatomical studies of PTSD brain, it will be important to factor in these overlying tissue aging effects into individual pathway and protein analyses.

BODY:

KEY RESEARCH ACCOMPLISHMENTS:

Project Specific:

Longitudinal study

Sample 2000 active duty/guard troops predeployment
Resample/test post-deployment

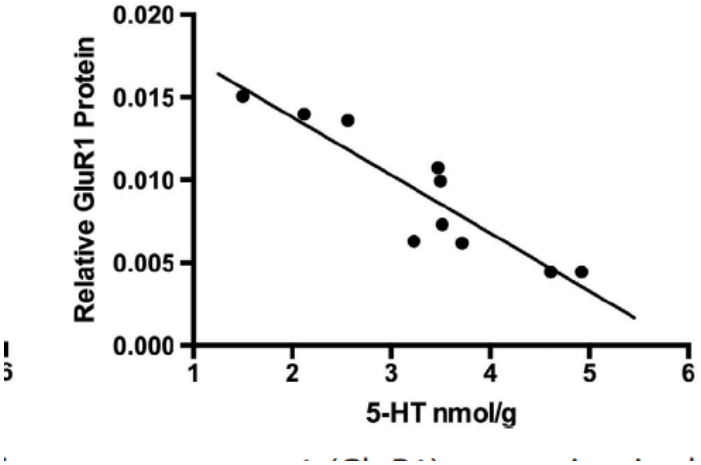
These tasks were rebudgeted due to lack of time to complete.

Pre-deployment/post-deployment MRI testing *300 scanning sessions*

This task was rebudgeted due to lack of time to complete the study and removal of the MRI scanning unit back to the factory.

Neurobiology

A. Animal model: Study the role of serotonin in subcortical fear signaling



Serotonin depletion increases Glutamate receptor density in the amygdala

Serotonin depletion modeling 5HTTLPR effects accentuates fear behavior

(abstract) Our previous experiments demonstrated that systemic depletion of serotonin (5-hydroxytryptamine, 5-HT), similar to levels reported in patients with emotional disorders, enhanced glutamateric activity in the lateral nucleus of the amygdala (LA) and potentiated fear behaviors. However, the effects of isolated depletion of 5-HT in the LA, and the molecular mechanisms underlying enhanced glutamatergic activity are unknown. In the present study, we tested the hypothesis that depletion of 5-HT in the LA induces increased fear behavior, and concomitantly enhances glutamate receptor (GluR) expression. Bilateral infusions of 5,7-dihydroxytryptamine (4 μg per side) into the LA produced a regional reduction of serotonergic fibers, resulting in decreased 5-HT concentrations. The induction of low 5-HT in the LA elevated fear-potentiated startle, with a parallel increase in GluR1 mRNA and GluR1 protein expression. These findings suggest that low 5-HT concentrations in the LA may facilitate fear behavior through enhanced GluR-mediated mechanisms. Moreover, our data support a relationship between 5-HT and glutamate in psychopathologies. (Attached) Tran, et al. 2013. Depletion of serotonin in the basolateral amygdala elevates glutamate receptors and facilitates fear-potentiated startle. Translational Psychiatry, e298; doi:10.1038/tp.2013.66 .

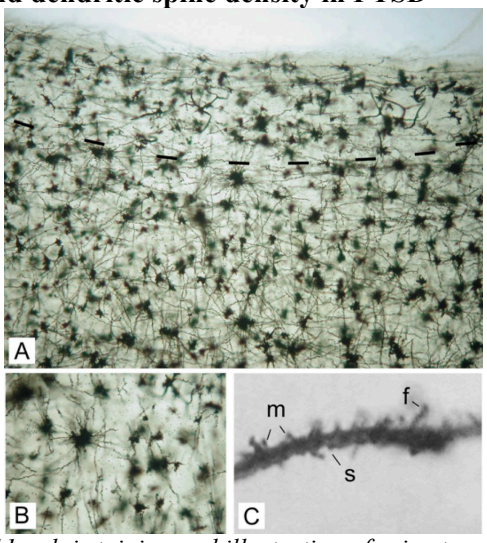
B. Collection of PTSD, MDD and control brains

PTSD cases contribute to new PTSD Brain Collection

Through our collection and phenotyping of over 40 PTSD and control cases, and by working with other brain banks to initiate brain collection, we can study at least 50 PTSD cases going forward for future studies. Initial studies on these specimens is underway as described below. Brains collected with current funding are being studied as part of a consortium with funding through the National Center of PTSD Brain Bank and the Consortium to Alleviate PTSD.

C. Compare gene expression in the frontal cortex of PTSD and controls.

Gene expression and dendritic spine density in PTSD



BA11 golgi staining and illustration of spine types

(abstract) *Genetic variants of the immunophilin FKBP5 have been implicated in susceptibility to post-traumatic stress disorder (PTSD) and other stress-related disorders. We examined the relationship between mushroom, stubby, thin and filopodial spine densities measured with Golgi staining and FKBP5 gene expression in the medial orbitofrontal cortex (BA11) in individuals diagnosed with PTSD and normal controls (n = 8/8). ANCOVA revealed PTSD cases had a significantly elevated density of stubby spines (29%, P < 0.037) and a trend for a reduction in mushroom spine density (25%, p < 0.082). Levels of FKBP5 mRNA were marginally elevated in the PTSD cases (z = 1.94, p = 0.053) and levels correlated inversely with mushroom (Spearman's rho = −0.83, p < 0.001) and overall spine density (rho = −0.75, p < 0.002) and directly with stubby spine density (rho = 0.55, p < 0.027). These data suggest that FKBP5 may participate in a cellular pathway modulating neuronal spine density changes in the brain, and that this pathway may be dysregulated in PTSD.* Young et al. 2015 BA11 FKBP5 expression levels correlate with dendritic spine density in postmortem PTSD and controls Neurobiology of Stress 2: 67–72

Gene expression changes in PTSD suggest accelerated aging/premature senescence.

We determined gene expression changes in BA11 (straight gyrus) in 18 PTSD and 36 control brains using the Infinium HumanMethylation450 BeadChip platform. 96% of the data passed initial QC checks. After variance stabilizing transformation and quantile normalization, we studied the top 30% most abundantly expressed transcripts in controls (13,600). These transcripts accounted for 92% of the dynamic range of expression levels in the sample. We then investigated 160 genes with with the greatest positive or negative fold-change from the pool of genes with nominally significant (P<0.05) changes in PTSD.

Reduced transcripts in PTSD: Topgene indicated that negative fold changes were enriched for multiple neuron-related GO categories, such as neuron projection morphogenesis. Note in the co-expression analysis (below), that more than one third of the reduced transcripts (63/160, FDR q< 2 x 10-56) overlapped with genes that are associated with interneurons, producing highly significant p and q values. Note also that the analysis indicates substantial enrichment in genes that are down-regulated in aging cortex (17/160, FDR q< 6.6 x10-13).

13: Coexpression [Display Chart] 5427 annotations before applied cutoff / 22914 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	M2110	Genes whose expression significantly and positively correlated with the density of CALB1-positive [GeneID=793] GABAergic interneurons in the BA9 brain region across all subjects with psychiatric disorders.	MSigDB C2: Broad Institute	3.780E-60	2.051E-56	1.882E-55	2.051E-56	63	548
2	M17728	Genes down-regulated in brain from patients with Alzheimer's disease.	MSigDB C2: University of Washington	1.174E-49	3.186E-46	2.924E-45	6.372E-46	73	1237
3	18223198-TableS3	Human Brain Chen-Plotkin08 747genes	GeneSigDB	3.568E-28	6.455E-25	5.924E-24	1.937E-24	40	594
4	M2105	Genes whose expression was significantly and positively correlated with the number of perineuronal oligodendrocytes in the layer III of BA9 brain region.	MSigDB C2: Broad Institute	3.244E-22	4.401E-19	4.039E-18	1.761E-18	38	756
5	M2107	Genes whose expression significantly and positively correlated with oligodendrocyte density in layer VI of BA9 brain region in patients with bipolar disorder.	MSigDB C2: Broad Institute	6.137E-17	6.661E-14	6.112E-13	3.330E-13	31	681
6	M9112	Age down-regulated genes in the human frontal cortex.	MSigDB C2: University of Washington	5.755E-16	5.206E-13	4.777E-12	3.123E-12	17	153
7	15720813-TableS2b	Human Brain Hoelzinger05 117genes	GeneSigDB	1.011E-11	7.837E-9	7.192E-8	5.486E-8	11	81
8	17676974-TableS1	Mouse StemCell Chambers07 1667genes	GeneSigDB	1.851E-11	1.256E-8	1.152E-7	1.005E-7	33	1236
9	M2892	Genes up-regulated in epithelial kidney cancer cell lines over-expressing an oncogenic form of KRAS [Gene ID=3845] gene.	MSigDB C6: Broad Institute	2.318E-11	1.397E-8	1.282E-7	1.258E-7	13	143
10	M8645	Genes up-regulated in the nucleus accumbens (a major reward center in brain) 2 weeks after induction of deltaFosB, a FOSB [GeneID=2354] splice variant.	MSigDB C2: University of Washington	3.951E-11	2.144E-8	1.968E-7	2.144E-7	9	48
11	M1712	Genes enriched in neurons in the adult mouse brain identified through correlation-based searches seeded with neuron cell-type specific gene expression patterns.	MSigDB C2: Broad Institute	4.655E-11	2.297E-8	2.108E-7	2.526E-7	10	69
12	M535	Genes up-regulated in CD34+ [GeneID=947] cells isolated from bone marrow of CML (chronic myelogenous leukemia) patients, compared to those from normal donors.	MSigDB C2: Broad Institute	7.849E-11	3.550E-8	3.257E-7	4.259E-7	34	1382
13	M2808	Genes up-regulated in neurons.	MSigDB C6: Broad Institute	1.055E-10	4.404E-8	4.041E-7	5.725E-7	11	100

Elevated transcripts in PTSD: Topgene indicated that positive fold changes were enriched for metalothionines (MT), with 6 of 10 MT transcripts represented in the top 160 positive fold changes (FDR q<6.7 x 10-11). In the co-expression analysis (below), 10% of the elevated transcripts (22/160, FDR q< 5 x 10-14) overlapped with genes that up regulated in aging.

13: Coexpression [Display Chart] 4928 annotations before applied cutoff / 22914 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	M5547	Age up-regulated genes in the human frontal cortex.	MSigDB C2: University of Washington	1.021E-17	5.030E-14	4.567E-13	5.030E-14	22	262
2	19843711-TableS2	Human Kidney Sallustio10 2134genes CompleteListAnalysis	GeneSigDB	2.032E-10	4.217E-7	3.829E-6	1.001E-6	37	1674
3	19843711-TableS1	Human Kidney Sallustio10 2134genes DiscriminatedARPCsFromRPTEC/MSC	GeneSigDB	2.567E-10	4.217E-7	3.829E-6	1.265E-6	37	1688
4	M1941	Genes with high-CpG-density promoters (HCP) bearing histone H3 dimethylation at K4 (H3K4me2) and trimethylation at K27 (H3K27me3) in brain.	MSigDB C2: Broad Institute	2.337E-8	2.833E-5	2.573E-4	1.152E-4	26	1069
5	18593933-Table1a	Human Lung Magda08 21genes	GeneSigDB	2.875E-8	2.833E-5	2.573E-4	1.417E-4	5	14
6	16293578-SuppTable2	Human StemCell Cai06 1370genes	GeneSigDB	1.939E-7	1.592E-4	1.446E-3	9.554E-4	26	1190
7	M2806	Genes up-regulated in astrocytes.	MSigDB C6: Broad Institute	4.881E-7	3.436E-4	3.120E-3	2.405E-3	8	100
8	16288205-GeneTable2	Human Breast Charafe-Jauffret06 1233genes	GeneSigDB	8.580E-7	5.285E-4	4.799E-3	4.228E-3	20	807
9	M17572	Genes down-regulated in the luminal B subtype of breast cancer.	MSigDB C2: Broad Institute	2.000E-6	1.058E-3	9.604E-3	9.854E-3	16	562
10	18459106-Table3	Human Viral Prueitt08 34genes	GeneSigDB	2.218E-6	1.058E-3	9.604E-3	1.093E-2	5	31
11	M1784	Genes changed in U373-MG cells (malignant glioma) upon treatment with arsenic trioxide [PubChem=14888], a chemical that can cause autophagic cell death.	MSigDB C2: Broad Institute	2.361E-6	1.058E-3	9.604E-3	1.163E-2	8	123
12	18310505-TableS8	Human StemCell Matushansky08 297genes	GeneSigDB	2.968E-6	1.151E-3	1.045E-2	1.463E-2	11	269
13	M4196	Genes up-regulated in PC3 cells (prostate cancer) after knockdown of EZH2 [GeneID=2146] by RNAi.	MSigDB C2: Broad Institute	3.036E-6	1.151E-3	1.045E-2	1.496E-2	22	1037
14	M13014	Genes down-regulated in liver tumor compared to the normal adjacent tissue.	MSigDB C2: Broad Institute	3.542E-6	1.201E-3	1.090E-2	1.746E-2	11	274

We have performed several error-checking routines to validate the above primary findings, including using random gene lists from the 13.6k expression database (background) in Toppgene and perfoming DAVID enrichment analysis against the 13.6 background. Specifically, in the co-expression lists above, some items (such as M535 = CD34+ related) registered as moderately enriched with random gene lists picked from the

background of 13.6k genes. This was never the case for positive (M5547), negative (M9112) age-related sets, the calbindin–positive set (M2110) or metalothionines. Finally, we repeated the analysis using all genes that passed QC (rather than those in the top 92% of the dynamic range), and found that both positive, negative and calbindin sets remained significantly enriched after bonferroni correction. These analyses support the major findings of the gene expression analysis implicating accelerated aging and deficits in interneuron transcripts in PTSD.

Overall, the gene expression data to date suggest that there are strong signs of accelerated aging, or premature senescence in PTSD frontal cortex, possibly accompanied by changes in cell populations similar to that observed in normal tissue aging. This is entirely consistent with our finding (C above) of reduced dendritic spine density in BA11 in PTSD, also a sign of aging. It is worth noting that while our PTSD cases were not significantly different in mean age compared to the controls cases (p=.05), the mean age was actually less in the PTSD group (43.1± 3.3SEM vs 47.6±2.2SEM). Premature senescence, reported in many tissues in the body in PTSD, will likely be linked to disruptions in the autonomic nervous system and HPA axis. We are preparing this data for publication.

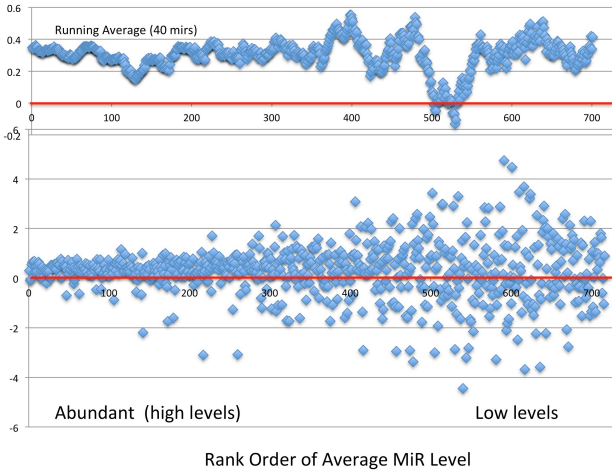
D. Compare anatomical markers in PTSD, MDD and controls, with 5HTTLPR and other genetic variants as cofactors.

Molecular studies of microRNAs (miRs).

miRs in PTSD

We have performed MiRNA studies of BA11 in PTSD and found that approximately 1 in every 6 (112/716) brain-expressed miRs were significantly elevated in PTSD at a nominal p value of 0.05, while only 4 were significantly decreased. This is strong evidence ($t < 4.2 \times 10(-17)$, fisher's exact test) for a global up-regulation of miRs in PTSD. This increase was not linked to changes in DICER transcript levels. This data has been submitted for publication.

92 out the 100 most abundant miRs in Cortex are numerically elevated in PTSD (P<3.3 x 10(-11)



Serotonin transporter genetic effects on miRs

In a second set of findings, we observed evidence for systematic changes in miR populations in the mediodorsal thalamus (MD) due to 5HTTLPR genetic effects. The MD provides powerful glutamateric input to the frontal cortex and is an important node in limbic circuitry. MiR levels and 5HTTLPR genotype with rs23456 following the "triallelic" convention were assessed in RNA/DNA extracted from frozen MD specimens of the Stanley Foundation Research Consortium (12 MDD, 12 SKZ and 14 controls) using PCR and Exiqon Human miRnome V1.M RT-PCR panels, resulting in an N = 577 MD-expressed miRs. A common pattern was that 5HTTLPR SS alleles were associated with reduced miR levels compared to SL genotype across many miR species, as evidenced by a significant reduction in total miR load (p<0.027), controlling for diagnosis, age, gender and PMI.

The most reliably affected SL>SS miRs (abundant miRs with p's <0.02) included miRs-7d*, 1979, 320a, 432, 532-5p, 664 and 92b. Interestingly Mir-432, which targets ELK1 (a transcription factor binding to 5HTTLPR), has previously been found to be decreased in SKZ blood. The 7 SS-reduced miRs targeted PTPRD, CNIH, FAM19b, and TOB1 mRNAs as a group. Several of these genes have been previously associated with either SKZ (CNIH, TOB1 and PTPRD) or MDD (TOB1). MiR-377 had the most reliable evidence for elevated levels compared to SL/LL. Several miRs expressed at relatively low levels displayed significantly altered but skewed distributions suggestive of selective miR editing in SS or LL genotypes, with loss of probe annealing potentially due to edited miR sequences. There was no relationship between 5HTTLPR genotype and MD mRNA levels for DGCR8, Drosha or Dicer transcripts. The present data support a pathophysiological phenotype consisting primarily of reduced miR levels in the MD nucleus of the thalamus associated with the 5HTTLPR-SS genotype. We are preparing this data for submission.

Serotonin transporter genetic effects on serotonin innervation

In addition to affecting uptake of serotonin on a real-time basis by changing transcription efficiency, the serotonin transporter promoter region (5HTTLPR) long/short genetic variation may influence regulation of developmental changes in 5HTT. We have investigated the influence of 5HTTLPR on serotonin innervation patterns in the anterior nuclei of the thalamus, an area involved in delivering sensory information from the environment to the amygdala. Serotonin is an important factor in determining both synaptic and morphological plasticity, and reducing serotonin transport levels during development results in permenant alterations in thalamic anatomy. We used sterelogical techniques to measure the density of serotonin fibers in the anterior thalamus and found a greater than 50% reduction in fiber denstity in SS cases compared to L carriers ($t=2.538$, Fratio = 6.45, DF=1 DFDEN=8.43, $p<0.0334$). This finding suggests that either we could not see some serotonin fibers in SS cases (because 5HTT protein synthesis was turned off) or that there was a change in how serotonin innervation developed in the anterior thalamus. This data is being prepared for publication.

REPORTABLE OUTCOMES:

L Tran, BK Lasher, KA Young and NB Keele (2013) Depletion of serotonin in the basolateral amygdala elevates glutamate receptors and facilitates fear-potentiated startle. Translational Psychiatry3, e298; doi:10.1038/tp.2013.66 (Appendix 1)

KA Young, P Thompson , D Cruz, DC Williamson, LD Selemon (2015) BA11 FKBP5 expression levels correlate with dendritic spine density in post-mortem PTSD and controls. *Neurobiology of Stress* 2: 67-72. (Appendix 2)

CONCLUSION: This research is continuing with funding through W81XWH-11-2-0166. The present data summarizing work from W81XWH-07-1-0244, suggests that like other organs, the brain in PTSD may be experiencing premature senescence, and future studies of biomarkers will need to take this into account as individual pathways are singled out for detailed study.

APPENDICES:

A. Tran et al., 2013 (P 12-19)
B. Young et al., 2015 (P 20-25)

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BA11 FKBP5 expression levels correlate with dendritic spine density in postmortem PTSD and controls

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ABSTRACT

Genetic variants of the immunophilin FKBP5 have been implicated in susceptibility to post-traumatic stress disorder (PTSD) and other stress-related disorders. We examined the relationship between mushroom, stubby, thin and filopodial spine densities measured with Golgi staining and FKBP5 gene expression in the medial orbitofrontal cortex (BA11) in individuals diagnosed with PTSD and normal controls (n = 8/8). ANCOVA revealed PTSD cases had a significantly elevated density of stubby spines (29%, $P < 0.037$) and a trend for a reduction in mushroom spine density (25%, $p < 0.082$). Levels of FKBP5 mRNA were marginally elevated in the PTSD cases ($z = 1.94$, $p = 0.053$) and levels correlated inversely with mushroom (Spearman's rho = −0.83, $p < 0.001$) and overall spine density (rho = −0.75, $p < 0.002$) and directly with stubby spine density (rho = 0.55, $p < 0.027$). These data suggest that FKBP5 may participate in a cellular pathway modulating neuronal spine density changes in the brain, and that this pathway may be dysregulated in PTSD.

1. Introduction

The medial orbitofrontal cortex (mOFC, Brodmann Area 11, BA11) is an integral hub of the limbic system which has been hypothesized to play an important role in depression and PTSD. The mOFC, together with the amygdala and mediodorsal thalamus, are critical for the management of emotional learning and for linking this learning with visceromotor responses. As outlined by Ongur and Price (2000), the mOFC, driven by inputs from the subgenual anterior cingulate cortex, integrates information from the limbic cortex, amygdala and mediodorsal thalamus and provides output to the striatum and brainstem/hypothalamus that can affect physiological systems such as heart rate and cortisol release (Angrilli et al., 2007; Eippert et al., 2007). Loss of plasticity in the mOFC could "lock" this pathway in the "on" position, preventing the brain from disengaging visceromotor responses to emotional stimuli, thus contributing to the maintenance of excessive physiological responding in posttraumatic stress disorder (PTSD).

Study of the relationship between brain cellular architecture and acquired response to stress has been investigated primarily in animal models. Severe stress is associated with both short-term and long-term changes in the central nervous system (CNS) and hypothalamic-pituitary-adrenal (HPA) axis, including alterations in dendritic spine architecture. For instance, after chronic restraint stress in mice, up to a 60% reduction in spine density in the anterior cingulate cortex can occur (Kassem et al., 2013). Acute predator stress in rodents, thought to have relevance to PTSD because it generates long-lasting hyperarousal and anxiety, has also been associated with decreased spine density in the hippocampus 16 days after a single traumatic exposure (Adamec et al., 2012). Cellular pathways that are involved in synaptic plasticity and remodeling may thus contribute to long-term changes in dendritic morphology that are likely to represent a final common pathway in PTSD for the behavioral persistence of PTSD symptoms.

FK506-Binding Protein 5 (FKBP5) is a gene that has been implicated in enhanced susceptibility to PTSD and depression

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(Szczepankiewicz et al., 2014; Boscarino et al., 2011). One possible mechanism contributing to this susceptibility is FKBP5's role in mediating glucocorticoid receptor turnover and sensitivity in the brain. High levels of FKBP5 in the brain can suppress glucocorticoid receptor-mediated enhancement of synaptic plasticity, and this action has been shown to have detrimental effects on dendritic architecture (Bennett and Lagopoulos, 2014). In addition to observing changes in dendritic spine density in PTSD, we observed a strong relationship between FKBP5 transcript levels and spine density, suggesting that this gene may play a role in mediating cell regulatory responses that are altered in PTSD.

2. Methods

2.1. Postmortem collection

Postmortem brain tissue from normal control (n = 8) and PTSD (n = 8) subjects was obtained from the Southwest Brain Bank (SWBB), Department of Psychiatry, University of Texas Health Science Center at San Antonio (UTHSCSA), with consent from the next-of-kin (NOK). The SWBB postmortem brain tissue collection operates under the jurisdiction of the State of Texas Anatomical Review Board and the UTHSCSA IRB oversees the interviews with the NOK. Trained clinicians interview the NOK about the donor and conduct a proxy DSM-IV based Mini-International Neuropsychiatric Interview (MINI). Diagnoses were determined by expert diagnostician group consensus (MINI inter-rater reliability = 0.8) after review of the interview materials and available medical records (Table 1). Both gross and microscopic neuropathology exams were conducted by a neuropathologist and all specimens were found to be free of confounding neuropathology. All analyses were performed in JMP 10.0.0 (SAS Software, Cary NC). Work was performed under the supervision of the Yale, UTHSCSA and Texas A&M University IRBs.

2.2. Golgi analysis

Blocks of fresh frozen tissue from area BA11 were coded and all analyses were performed blind to diagnosis. Blocks were thawed overnight in 4% paraformaldehyde fixative and then processed with the FD Rapid GolgiStain™ kit as follows. Blocks were immersion fixed for approximately 2 weeks in a combination of solutions A (Potassium dichromate and mercuric chloride) and B (Potassium chromate) followed by one week of cryoprotection in solution "C" (proprietary recipe). Thick sections (200 μm) were cut on a Vibratome and mounted on gelatin-coated glass slides. The sections were treated with solutions D and E (proprietary recipe) for signal development before being dehydrated through graded alcohols and cover-slipped with Permount.

A sampling strategy based on the fractionator method developed for stereologic analysis (Gundersen et al., 1988) was employed; note however that the entire expanse of BA11 was not available for analysis as is required for truly unbiased sampling. Contours were drawn around the cortex in BA11 from the pial surface to the subjacent white matter and therefore including all six cortical layers in 3–5 sections of each brain. With the aid of a MicroBrightField Neurolucida system (Williston, VT), the fractionator sampling method was applied by placing a grid over the contour with counting frames randomly placed in such a manner that 20 sampling sites were generated for each brain area. All visible spine heads protruding from the shaft were counted along 20 μm-long dendritic segments in the counting frames (Fig. 1). Spines were classified into the following categories as described by Bourne and Harris (2007): thin, mushroom, stubby, filopodial and unclassified. Spine density was calculated as number of spines per micron.

2.3. FKBP5 analysis

FKBP5 mRNA was extracted from adjacent blocks of frozen BA11 using Qiagen RNeasy Lipid Tissue Mini Kit and stored at −80°. Whole genome expression analysis using Illumina Human WG-6 v3.0 Expression BeadChips (Illumina Inc, San Diego CA) "direct hybridization" assay was performed on blinded specimens. Arrays were scanned on an Illumina iScan System™ and processed on the Illumina GenomeStudio software. mRNA levels of FKBP5 in all cases were detectable with $p < 0.0001$.

3. Results

3.1. Spine density changes in PTSD

Approximately 500 spines were counted per brain (range 382–654). Only 1.39% of all observed spines could not be classified. In control brains, mushroom shaped spines accounted for 57% of the spines in BA11, followed by stubby (18%), thin (18%) and filopodial spines (7%). Shapiro–Wilk W test indicated that all sets of spine density data were normally distributed. ANCOVA's (two-tailed, DF = 1,15) with diagnosis, age, gender, PMI and pH as covariates revealed that PTSD cases had significantly elevated density of stubby spines (29%, PTSD LSMean [SE] = 0.212 [0.031], control = 0.299 [0.033], $p < 0.037$), and there was a trend for a decreased density of mushroom spines in these cases (25%, PTSD = 0.813 [0.090], control = 0.612 [0.010], p = 0.082). The stubby spine density finding did not survive Bonferroni correction.

3.2. FKBP5 levels and spine density

Shapiro–Wilk W test indicated that the FKBP5 data was not normally distributed. A Median Test found that levels of FKBP5 mRNA were borderline elevated in the PTSD cases (DF = 14, z = 1.94, p = 0.053). FKBP5 levels were inversely correlated to total spine density (Spearman's rho = −0.75, $p < 0.002$) and mushroom spine density (Spearman's rho = −0.83, $p < 0.001$ (Fig. 2)) and directly correlated with stubby spine density (Spearman's rho = 0.55, $p < 0.027$). FKBP5 correlations with mushroom and total spine densities survived Bonferroni correction.

3.3. Clinical confounds

Smoking is a common confound in many post-mortem studies because of its high prevalence in psychiatric conditions. All PTSD cases had a history of smoking, while only 50% of the controls were smokers. Comparing smoking vs non-smoking controls, we did not observe any effect of smoking on spine density or FKBP5 levels (DF = 7, all t-test p's > 0.34). None of the controls had a history of heavy drinking, while a history of heavy alcohol use was common in the PTSD group (6/8), and it was not not possible to examine effects of this potential confound in the present data set.

4. Discussion

In the present study, we tentatively identified differences in dendritic spine architecture in samples of BA11 obtained from the frontal cortex of people who were affected by PTSD. An increase in density of stubby spines in BA11 of PTSD subjects was accompanied by trend reduction in the density of mushroom spines. These findings did not survive Bonferroni correction, and they will need to be validated in a larger sample. Levels of FKBP5 were significantly correlated with these changes, with the results being consistent with animal model data indicating that elevated levels of

Table 1
Demographics.

	PTSD (n = 8)	Control (n = 8)	p for 2-sided t-test^ or
	Mean ± SEM	Mean ± SEM	Fisher's Exact Test^^
Age (years)	53.0 ± 4.7	60.0 ± 3.2	0.2380^
Gender (M/F)	7/1	7/1	–
Race (W,H,AA)	(4,3,1)	(4,4,0)	–
Hemisphere (R/L)	7/1	7/1	–
PMI (hours) ^	24.1 ± 2.0	24.4 ± 2.8	0.9338
Tissue pH ^	6.43 ± 0.10	6.22 ± 0.70	0.4102
Smoking History ^^	8	4	0.0769
Alcohol Use (heavy/light) ^^[a]	8 (6,2)	2 (0,2)	0.0070
Antidepressant History^^	7	0	0.00007

M = Male, F = Female, W = White, H = Hispanic, AA = African American.
[a] Comparing cases with any alcohol history: PTSD = 8/8, controls = 2/8, DF = 14 for all comparisons.

FKBP5 are associated with impaired synaptic function and increases in stress behaviors.

The observed alterations in spine density in PTSD are consistent with a regression of cortical synaptic architecture in BA11 to a less mature state (Harris et al., 1992). Some mature mushroom spines appear to have been replaced by simpler, stubby spines. Mushroom spines have constricted spine necks that act to contain the rise in calcium levels generated by synaptic activity locally within the spine head (Yuste et al., 2000). In contrast, stubby spines provide a relatively unrestricted path for calcium diffusion from the spine to the dendritic shaft due to the absence of the spine neck. Local concentration of calcium in the spine head creates an environment that is conducive to LTP-induced plasticity (Malenka et al., 1988; Holmes, 1990). Thin spines are thought to take part in plasticity-mediated learning and, under conditions that generate LTP, can enlarge into more stable mushroom spines believed to be important for long-term storage of memory (Kasai et al., 2003; Bourne and Harris, 2007). The enlarged head of a mushroom spine contains a large post-synaptic density, a spine apparatus and associated spine organelles, all important components of calcium regulation that are not found in other spine types including stubby spines (Sorra and Harris, 2000; Bourne and Harris, 2007). Thus, although the details of stubby spine physiology have yet to be fully established, most of the available data suggests that stubby spines in the mature brain would have a reduced capacity to make contributions to synaptic adaptability (i.e., LTD and LTP). Our finding provides evidence of a structural variation that could contribute to reduced plasticity in the mOFC in PTSD, making this area less capable of synaptic learning. New learning via LTP is thought to underlie conditioned fear extinction, the ability to quench adverse reactions to previously experienced dangerous situations that are no longer a threat, and failure to extinguish conditioned fear is the cardinal symptom of PTSD (Quirk et al., 2006).

One important observation is that the shift from mushroom to stubby spine morphology means that there may be reduced numbers of spine necks in PTSD. The constricted neck of a mushroom spine has multiple functional roles. In addition to affecting calcium signaling, spine necks may prevent the exodus of AMPA receptors from the spine head, thereby maintaining the strength of the synapse (Kusters et al., 2013). The spine neck also is an important site for shaping incoming excitatory signals passing from the PSD into the dendrite. For instance, dopamine and noradrenergic receptors have been localized to spine necks (Smiley et al., 1994; Bergson et al., 1995; Wang et al., 2007). The latter have also been shown to colocalize with hyperpolarization-activated cyclin nucleotide-gated (HCN) channels in the spine neck of long-thin spines (Wang et al., 2007; Paspalas et al., 2013), suggesting that spine necks are important for momentary changes in activation of prefrontal network activity associated with arousal, motivation and stress, as well as potentially for longer term effects mediated by LTP induced plasticity (Arnsten et al., 2010).

Alterations in dendritic spines have been found in association with neuropsychiatric illness. For example, using a normal, non-psychiatric human cohort and non-Golgi molecular techniques, Soetanto et al., 2010 found that psychological stress was associated with spine changes in the hippocampal CA3 region. Individuals reporting high levels of anxiety and depression had reduced density of both dendrites and spines in this area. In schizophrenia, robust reductions in dendritic spine density have been reported on pyramidal neurons in layer V and III of the frontal cortex (Broadbelt et al., 2002; Glantz and Lewis, 2000; Garey, 2010), subiculum (Rosoklija et al., 2000) and temporal cortex (Garey et al., 1998). In MDD, spine density was found to be reduced in the prefrontal cortex (Kang et al., 2012). These studies suggest that spine density changes may be a common final pathway of neuronal pathophysiology in several psychiatric conditions.

Dendritic spine alterations have been observed in a variety of animal models of potential relevance to PTSD. For instance, repeated restraint stress in rats alters spine morphology in the medial prefrontal cortex, resulting in fewer large spines (Radley et al., 2008). A chronic variable stress regimen in rats decreases spine density on pyramidal neurons in the prelimbic cortex and selectively reduces mushroom spines on neurons in the anterior bed nucleus of the stria terminalis (Radley et al., 2013). In this later model, animals displayed attenuated functional activation of prelimbic cortex neurons projecting to the anterior bed nucleus of the stria terminalis, which is an important center providing inhibitory control over the HPA axis. After chronic variable stress, these animals responded to an additional stressor by hyperactivation of HPA

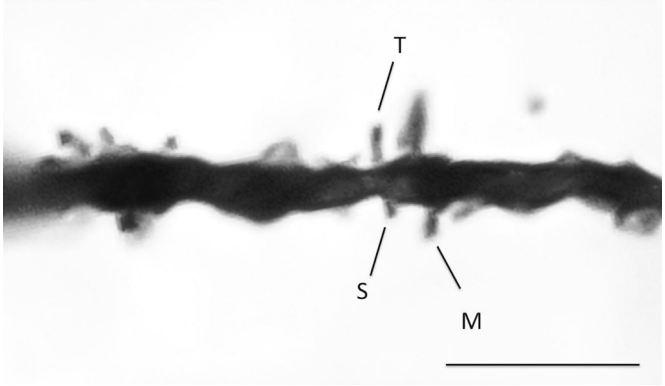

Fig. 1. Representative dendrites from a control brain are shown at 63× magnification. Stubby (S), Thin (T) and Mushroom (M) spines are labeled in this example. Scale bar = 5 μm.

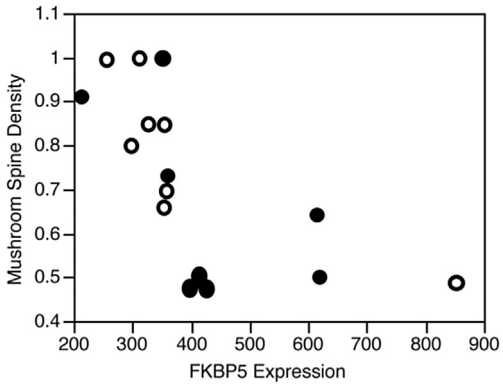

Fig. 2. FKBP5 gene expression levels in BA11 were inversely correlated to mushroom spine density (Spearman's rho = −0.83, $p < 0.001$) in PTSD (filled) and control cases (unfilled).

axis-related products such as plasma cortisol. One interesting observation is that animals susceptible to chronic defeat stress, in addition to having more stubby spines in the nucleus accumbens, display reduced post-synaptic density area and altered electrophysiology (Christoffel et al., 2011). Although miniature excitatory post-synaptic (mEPSC) amplitude was not changed, the frequency of mEPSCs was more than 50% higher in neurons with elevated numbers of stubby spines (Christoffel et al., 2011). These stress-related spine morphology changes were correlated with behavioral changes such as loss of social interactions and anhedonia-like behavior. Additional work is needed to determine whether changes in dendritic spine density in BA11, such as observed in our study, may contribute to long-term changes in HPA axis function in PTSD.

The pattern of reduced density of mushroom spines coupled with elevations in stubby spines has been reported for various stressors. For instance, in hippocampal culture, knockdown of SNX26, a synapse-related guanosine triphosphatase, significantly increased stubby spines at the expense of mushroom spines (Kim et al., 2013). In the hippocampus of adolescent rats, MK-801 reduced the density of mushroom spines and increased the density of stubby spines (Han et al., 2013). Finally, in the motor and somatosensory cortex of a model of Downs syndrome, mice had significantly fewer mushroom spines and significant increases in the number of stubby spines (Haas et al., 2013). Several animal models have thus found increases in stubby spines accompanied by reduced density of mushroom spines.

The mechanisms influencing the apparently enduring changes in OFC spine morphology are not well understood. One possibility is that the observed changes in spine architecture are driven by excessive glutamatergic drive through the OFC during the development of PTSD, and that the system has responded by reducing spine density, which would limit synaptic area available for excitatory signaling. The ability of ketamine (glutamatergic NMDA antagonist) to reverse spine loss in the prefrontal cortex of animal models of depression support this possibility (Nanxin et al., 2010). Furthermore, a recent MRI study provides support for loss of neuropil in the OFC in PTSD. Using pre and post-trauma MRI's, Sekiguchi et al. (2013) found that the OFC was the only region in the brain where PTSD symptoms were significantly correlated with grey matter volume reductions. In mice, stress has been observed to reduce grey matter volumes assessed with MRI, and anatomical studies have confirmed that this reduction is correlated with attenuation of alterations in dendritic spines (Kassem et al., 2013).

In addition to the association of FKBP5 polymorphisms with susceptibility to PTSD and depression (Szczepankiewicz et al., 2014; Boscarino et al., 2011), FKBP5 expression has been reported to be elevated in the cortex in individuals with depression (Sinclair et al., 2013). FKBP5 has been shown to be associated with impairments in stress resiliency and altered glucocorticoid signaling in rodent models (Scharf et al., 2011; Touma et al., 2011). Given that glucocorticoid receptor signaling is known to play an important role in mediating synaptic plasticity (Sarabdjitsingh et al., 2014), the present data supports the possibility that FKBP5 impacts synaptic processes through its relatively well-characterized interactions with glucocorticoid receptor signaling in neurons. However, research on FKBP5 is in its infancy, and this immunophilin has been linked to other cellular pathways and it is active in several cell types including glia. In astrocyte cell cultures, for instance, glucocorticoids induce a 2-fold elevation in FKBP5 levels (Carter et al., 2012). In its role as a molecular chaperone, FKBP5 participates in the construction and maintenance of the RISC complex that mediates microRNA processing (Martinez et al., 2013), and it is also involved in folding the microtubule associated protein Tau in Alzheimer's disease (Koren et al., 2011). Finally, it is worth noting that while FKBP5 may be present in elevated levels in brain in PTSD, several studies have indicated that reduced levels are present in blood, and that this may be associated with a long-term up-regulation of glucocorticoid sensitivity in blood cells (Yehuda et al., 2009; Sarapas et al., 2011). It is likely that many gene transcripts other than FKBP5 will be found to be correlated with spine density, however, the interesting biological phenotypes of FKBP5 knock out models (stress resilience and learning potentiation) argue for the possibility that this gene is a critical mediator of synaptic changes in the brain in stress-related conditions. If this is the case, then agents that can normalize FKBP5 levels in the brain may prove to be effective treatments for PTSD and other stress-related disorders.

The present study illustrates some of the strengths of human post-mortem studies in identifying initial pathophysiological pathways that deserve additional investigation in understanding the brain's response to stress. However, it should be considered that human post-mortem studies are commonly affected by multiple confounds, as was the case in the present study. This includes medication (7/8 antidepressant usage in PTSD, 0/8 in controls) and elevated rates of substance abuse and smoking in PTSD. It should also be remembered that there is abundant evidence for a substantial biological overlap between MDD and PTSD, and therefore, the present findings need to be further studied to determine whether they represent a shared pathophysiology, or to what extent changes may be limited to PTSD. Some of the variation observed in this study could be due to natural causes related to psychiatric condition at the time of death. If spine density changes are trait markers, it would be expected that theses changes might be exacerbated in individuals who were experiencing an episode of PTSD at the time of death. In the future, it will be interesting to study other brain regions to determine whether the observed findings are localized to frontal cortex or whether they represent a widespread phenomenon in PTSD. In one of the only other studies of post-mortem tissue in PTSD (Su et al., 2008), researchers focused on mitochondrial gene expression and did not measure spine density, so it will also be interesting to ascertain whether the mitochondrial pathways identified as abnormal in that study might be related to the changes in FKBP5 levels observed in this study.

In this first study of dendritic spine architecture in PTSD, the small number of specimens limits available power to withstand multiple comparison statistics with the PTSD phenotype, and replication will be important. However, the strong correlation of dendritic spine density to FKBP5 suggests that study of individual cellular pathways may provide a powerful tool for understanding the pathophysiology of stress in future studies of PTSD and other stress-related conditions.

Authors contributions

The authors equally shared in the design, implementation and analysis of the work. KAY wrote the initial draft of the manuscript and all authors shared in editing the manuscript.

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